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Attempts to Improve Survival of Neurons Derived from Neonatal Rat Hypothalamus-Preoptic Area in Serum-free Media

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ABSTRACT—In primary culture of cells derived from neonatal rat hypothalamus-preoptic area, a great decrease in the survival of neurons following an increase in the number of non-neuronal cells was observed in serum-supplemented medium. However, the addition of cytosine arabinoside, a mitotic inhibitor, to serum-supplemented medium significantly enhanced the survival of neurons. In order to diminish the proliferation of non-neuronal cells, attempts were made to maintain dissociated cells in serum-free medium. The maintenance of neurons in healthy condition was very difficult in serum-free medium from the very beginning of cultivation. Culture in serum-supplemented medium followed by culture in serum-free medium was much superior to the culture in serum-free medium for the entire course of cultivation with regard to survival. In the next experiment, the effects of glass surface and poly-lysine-coated surface were compared. Cultured cells in serum-free medium on glass surface were better in morphology compared with those on poly-lysine-coated surface. In the latter, strange contraction of basal cell sheets disturbed long-term cultivation. The best culture condition established in the present study, i.e., preculture in serum-supplemented medium and then transfer to serum-free medium on glass surface substrate, was employed for the study on the effects of hydrocortisone. Survival rate of neurons was improved in cultures with hydrocortisone at the concentration of 10^{-10} M.

INTRODUCTION

The serum contains various factors which generally afford cultured cells a favorable condition. However, the composition of serum is variable, depending on the animals species, age and sex of its donor, and even on lots [1–3]. Moreover, in primary culture of the cells from the central nervous system using serum-supplemented medium, the overgrowth of non-neuronal cells makes the interpretation of experimental results difficult [4, 5]. To solve these problems, efforts have been exerted to replace serum by certain chemically defined agents [6, 7]. In the culture of neural cells, first breakthrough was made on the culture of clonal cell lines. Mather and Sato [8] tested effects of various factors on mouse melanoma M2R cultured in serum-free medium, and established favorable hormone supplements. Bottenstein and Sato [9] succeeded in culturing rat neuroblastoma line B104 in serum-free medium with supplements called "N2". Since then, studies have been carried out on neural cell cultures in serum-free medium and many investigators have pointed out that serum-free medium is beneficial for neuronal survival, but it is difficult to culture neural cells completely in serum-free medium [10, 11].

The aim of the present study was to examine the effect of inhibition of non-neuronal cell growth by the application of cytosine-β-D-arabinofuranoside (AraC), a mitotic inhbitior, on the survival of neurons, and to afford further evidence for the effects of serum, substrates and hydrocortisone supplementation for the primary culture of cells derived from neonatal rat hypothalamus-preoptic area (Hyp-POA) in order to establish a culture condition with serum-free medium. Cells derived from neonatal rat cerebral cortices were also used in some experiments for comparison.

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MATERIALS AND METHODS

Cell dissociation

Neonatal rats of the Wistar/Tw strain of both sexes were used. On the day of birth animals were decapitated, and the Hyp-POA and cerebral cortex were immediately taken out and pooled in a petri dish containing cold serum-supplemented medium. Tissue pieces were minced by fine scissors, and after discarding the medium, they were incubated with 1,500 PU/ml dispase (Gohdoh-shusei Co., Tokyo) in serum-supplemented medium for 15 min at 37°C. After incubation, they were rinsed twice with fresh cold serum-supplemented medium, followed by gentle pipetting in serum-supplemented medium to yield dissociated cell suspension. Remaining tissue pieces were then rinsed twice with Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS(−)) that contained 0.5% bovine serum albumin and 0.5% glucose (PBS(−)+BG). Then, they were incubated with 2 mM glycoletherdiaminetetraacetic acid (EGTA) in PBS(−)−BG for 15 min at 37°C and after twice washing with PBS(−)+BG, they were subjected to gentle pipetting in PBS(−)+BG to yield cell suspension. Both dispase and EGTA treatments were repeated for a few times. Smaller diameters of pipettes were used as the size of remaining tissue pieces became smaller. The cell suspension was passed through a nylon mesh (50 μm in pore size; NBC Industry Co., Tokyo) to remove large cell clumps, and centrifuged at 250× g for 5 min at 4°C. Cell pellets were resuspended in a fresh serum-supplemented medium and viable cells were counted by trypan blue exclusion test using a hemocytometer. Initial cell viabilities of the Hyp-POA and cerebral cortical cell suspensions were about 80% and 90%, respectively. The number of viable cells obtained from one pup was $4.5–6.0\times10^5$ for the Hyp-POA and $2.0–3.0\times10^6$ for the cerebral cortex. After dilution, the cells were plated on a round coverslip (14 mm in diameter) in multiwell culture plate (Sumitomo Bakelite Co., Tokyo). In some cases, the coverslip had been coated with certain substrates. Plating density was 1.5×10^5 cells/well for the Hyp-POA and 3.0×10^5 cells/well for the cerebral cortex. Unless otherwise stated, culture medium was renewed at 3-day intervals.

Culture media

Two types of serum-supplemented media were used. The first type consisted of 85% Eagle's minimum essential medium supplemented with 2 mg/ml sodium bicarbonate, 9.67 mg/ml glucose, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and of 15% fetal bovine serum (FBS). This medium (MEM-S) was used for culture in serum-supplemented medium.

Second type of serum-supplemented medium was used only for the preculture of serum-free culture. This medium (DME/F12-S) was composed of 15% FBS and 85% 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (EME/F12) supplemented with 5.4 mg/ml glucose, 1.2 mg/ml N-2-hydoxyethylpiperazine-N'-2-ethensulfonic acid (HEPES), 100 IU/ml penicillin and 100 μg/ml streptomycin.

For serum-free medium, the N2-supplements of Bottenstein and Sato [9] without progesterone were used, where DME/F12 with above-mentioned supplements without FBS was further added with 100 μg/ml human transferrin, 100 μM putrescine, 30 nM selenium (as Na_2SeO_3) and 5 μg/ml insulin. This serum-free medium was designated as DME/F12-F.

Insulin, putrescine, human transferrin and DME/F12 mixture with HEPES were purchased from Sigma. MEM was purchased from Nissui Phamaceutical Co. (Tokyo), and FBS from Hazleton Research Products Inc. (St. Louis).

Other chemicals

AraC (Sigma) at the concentration of 10^{-5} M was dissolved in MEM-S.

Hydrocortisone (Sigma) was first dissolved in ethanol and diluted in DME/F12-F at the final concentrations of 10^{-7}–10^{12} M in 0.007% ethanol.

Substrates

For poly-L-lysine coating, the method of Pettmann *et al.* [12] was used with a slight modification. Poly-L-lysine solution (500 μl, 100 μg poly-L-lysine per 1 ml boric acid buffer, pH 8.4) was

added on a coverslip placed in each well of culture plate for overnight at room temperature. After discarding the solution, the coverslip was rinsed twice with PBS(−). Poly-L-lysine (M.W. 52,000) was purchased from Sigma.

For serum precoating, the method of Eccleston *et al.* [11] was used with a slight modification. A coverslip was incubated with DME/F12-S for overnight. Before use, it was rinsed twice with PBS(−).

Cell counting

Cultured cells on the coverslips were fixed for 2 days in a fixative consisting of 70% ethanol, 5% neutral formaldehyde and 5% acetic acid. After about 1 week of washing in 99% ethanol, the cells were stained by a modified Bodian's method [13].

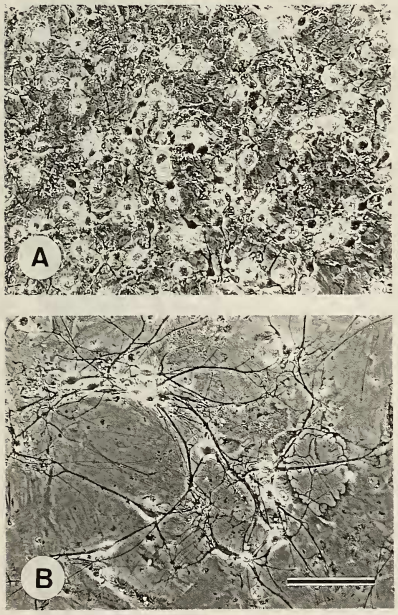
The cells which statisfied all the three following criteria in Bodian preparations were regarded as neurons; (i) the cell with positively stained cell body, (ii) the cell with strongly stained processes, and (iii) the cell at least one of the process length was more than 3-fold longer than the diameter of the cell body. The number of all the neurons on a coverslip or the number of neurons in randomly chosen 100 fields under a light microscope (one field equals to 0.19 mm^2) was counted.

RESULTS

Survival of neurons in serum-supplemented medium and effects of AraC

To examine the effects of non-neuronal cell population on neuronal survival in serum-supplemented medium, the Hyp-POA cells were cultured for the first 3 days in MEM-S on poly-lysine-coated surface, then the medium was replaced by MEM-S supplemented with or without 10^{-5} M AraC. After culture for 3 days with or without AraC, the cultures were washed twice with Ca^{2+}, Mg^{2+}-containing Dulbecco's phosphate buffered saline (PBS(+)), and the medium was replaced by MEM-S without AraC and the cells were continued to be cultured in MEM-S.

Phase-contrast photomicrographs of control



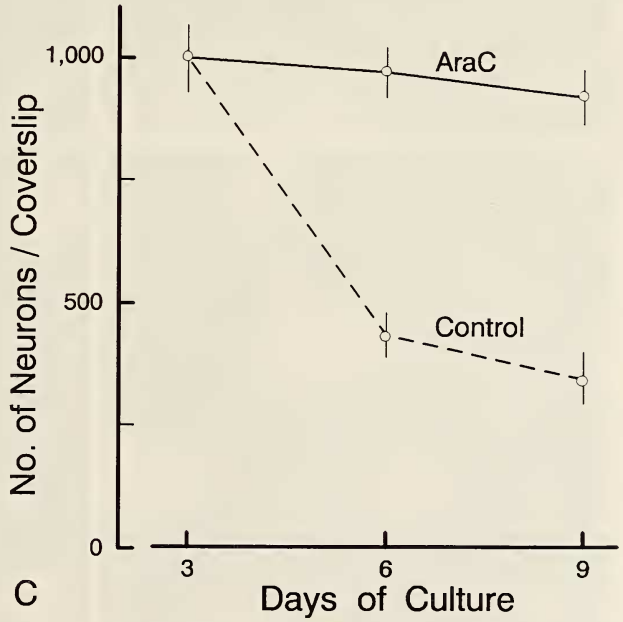


Fig. 1. Effects of AraC on Hyp-POA cell cultures for 13 days in MEM-S. A: Photomicrograph of control culture without AraC. B: Photomicrograph of AraC-added culture. Bar=100 μm. C: The number of neurons per coverslip at 3, 6, and 9 days of culture with (solid line) and without (broken line) AraC treatment. AraC treatment was performed between 3 to 6 days of culture. Vertical bars depict the standard errors of the means (n=2−4).

AraC-untreated cultures show that only few neurons were present, overlying the sheet of smaller cells on the substrate (Fig. 1A). Many glial cells, apparently dead cells (round brilliant cells) and few neurons were visible on the basal cell sheet. In AraC-treated cultures, non-neuronal cell proliferation was effectively inhibited and the basal surface was covered with larger flat cells. Many neurons survived on the basal sheet, and they extended prominent processes forming networks (Fig. 1B). Glial cells and apparently dead cells were less than the control AraC-untreated cultures.

Figure 1C shows the number of neurons per coverslip. In control cultures, the number of neurons greatly decreased at 6 days, while in AraC-treated cultures, the number of neurons was almost constant for 9 days, indicating that the proliferation of non-neuronal cells greatly disturbed the survival of neurons in serum-supplemented medium.

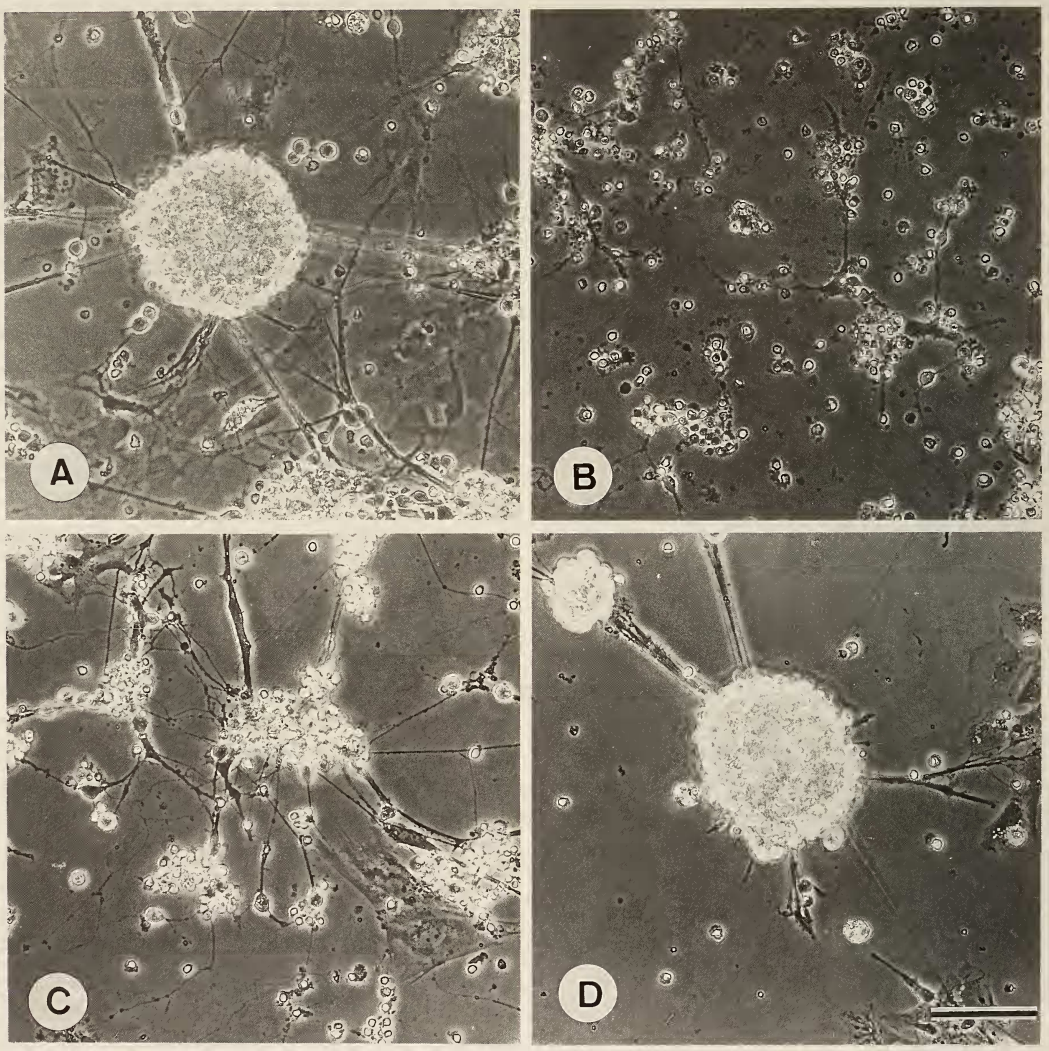


FIG. 2. Phase-contrast photomicrographs of cultured cells. A: Cells cultured for the first 2 days in DME/F12-S then 2 days in DME/D12-F. B: Cells cultured for the first 2 days in DME/F12-F then 2 days in DME/F12-F. C: Cells cultured for the first 2 days in DME/F12-F containing 0.5% BSA then 2 days in DME/F12-F. D: Cells cultured for the first 2 days in DME/F12-F on serum precoated surface then 2 days in DME/F12-F. Bar=100 μm.

Survival of neurons in serum-free medium and effects of substrate

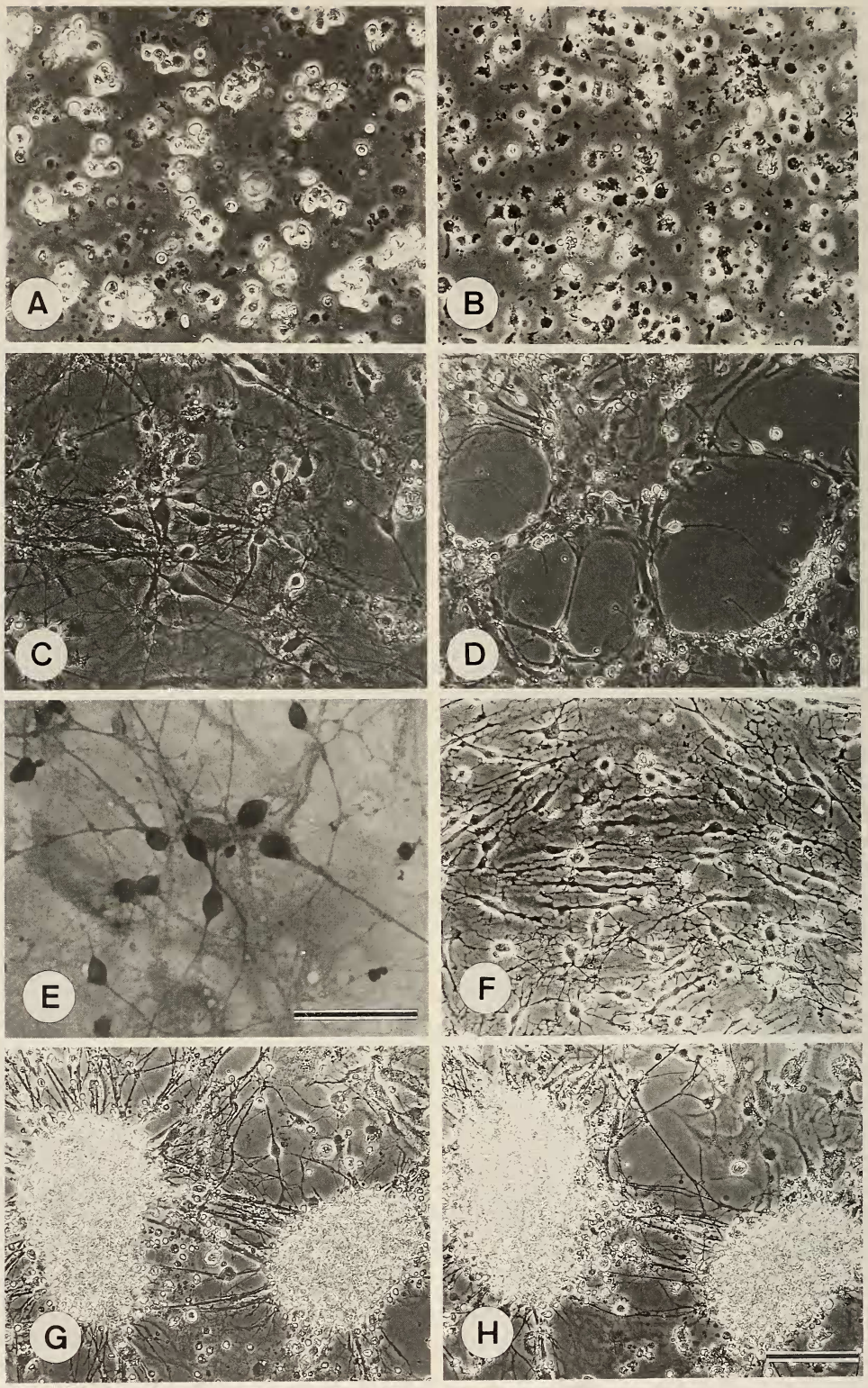
To maintain neuronal cells in serum-free medium throughout the culture period, some agents for cell protective and attachment-extension functions should be added to replace the serum components [14–16]. For this purpose the supplementation of 0.5% BSA to serum-free medium and serum precoating were separately tested. The cerebral cortical cells were used as test cells, and the fiber connections among aggregates were regarded as suitable indices for neuronal viability. After centrifugation of dissociated cell suspension, cell pellets were resuspended in DME/F12-S, DME/F12-F or DME/F12-F containing 0.5% BSA. The resuspensions in DME/F12-S and DME/F12-F containing 0.5% BSA were cultivated on glass surface. The cell resuspension in DME/F12-F was cultivated on glass surface or serum precoated surface. The medium of all the four groups was replaced by DME/F12-F after twice rinses with PBS(+) at 2 days of culture.

Figure 2 shows phase-contrast photomicrographs of cultured cells. When cells had been cultured in DME/F12-S before transfer to DME/F12-F, many cells survived and numerous fiber connections were formed (Fig. 2A). When they had been cultured in DME/F12-F from the very beginning of culture, there were few healthy aggregates, and fiber connections were not formed (Fig. 2B). Supplementation of BSA increased the survival of cells, while it had no appreciable effect on the formation of fiber connections (Fig. 2C). Serum precoating increased the formation of fiber connections, and it also had a slight beneficial effect on the survival of cells (Fig. 2D). When both supplementation of BSA and serum precoating were carried out, serum-free culture from the very beginning was considerably improved. However, it was still inferior to the culture with preculture for 2 days in serum-supplemented medium. Therefore, preculture in DME/F12-S for 2 days was used routinely for later experiments.

To compare culture conditions in serum-free medium, the Hyp-POA and cerebral cortical cells were cultured on glass or poly-lysine-coated surface in DME/F12-S for 2 days. Following the preculture, the medium was replaced by DME/F12-F.

On glass surface, cultured Hyp-POA cells first formed aggregates (40–100 μm in diameter) within 3–6 h (Fig. 3A). During preculture in serum-supplemented medium, the cells attached to glass surface and began to grow. Some neuron-like cells were present on extended non-neuronal cells or directly on glass surface, but most of them were observed to reside in the aggregates at 2 days of culture. Although non-neuronal cells continued to proliferate in serum-free medium, the growth was slower than that in serum-supplemented medium. As a consequence, basal sheet formation was delayed and the cells organizing basal sheet were larger and flatter than the cells in serum-supplemented medium. After reaching confluence, the overgrowth of non-neuronal cells was not so extensive and the translocation of glial cells from inside of the sheet to free surface was suppressed in serum-free medium. Neuron-like cells each possessing neurites, mostly bipolar, one clear ovoid nucleus and one or two nucleoli. Although moderate neural networks were observed before the completion of basal cell sheet formation, substantial network formation proceeded after the completion of basal sheet (Figs. 3C and E). The number of neurons was counted at 5, 8, 11, 14 and 17 days (Fig. 4). Because of numerous aggregates at 2 days of culture, the number of neurons was not counted. Basal cells became confluent between 8 to 11 days of culture. The number of neurons decreased at 11 days, and after that it remained unchanged. The Hyp-POA neurons could be maintained for more than 3 weeks in DME/F12-F on glass surface.

On poly-lysine-coated surface, individual cells adhered to the surface isolatedly (Fig. 3B). The developing pattern of the culture was generally similar to that of the culture on glass surface for several days. However, a drastic change began to occur during 8 to 14 days on poly-lysine-coated surface. Some basal cells began to detach from the surface and showed a change in morphology, from squamous to elongated shape. As a result of such transformation, many irregular-shaped uncovered surfaces appeared (Fig. 3D). Neural networks on this transforming basal sheet eventually dis-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appeared and some neuron-like cells left behind on the uncovered surface could not survive any longer. Some basal cells remaining on the uncovered surface proliferated to cover the surface again.

The cerebral cortical cells formed aggregates on glass surface, as the Hyp-POA cells did. At 2 days, fiber connections were formed among closely located aggregates. Although they had been cultivated at 2-fold higher cell density than the Hyp-POA cells, fewer cells could survive and settle during preculture. The proliferation of basal cells was very slow in serum-free medium and basal sheet failed to reach confluence even at 11 days. Few cells spread out from the aggregates of the cerebral cortical cells. Fiber connections among the aggregates increased for several days in serum-free medium, but they gradually degenerated (Figs. 3G and H). Most striking change in cerebral cortical cell culture was the appearance of non-neuronal cells bearing several processes with numerous branchings (Fig. 3F). This type of cells resembles differentiated astrocytes induced by glial maturation factor [26]. The appearance of differentiated astrocyte-like cells was less in Hyp-POA cultures than in cerebral cortical cell cultures in serum-free medium and was never observed neither in cerebral cortical nor Hyp-POA cultures in serum-supplemented medium.

On poly-lysine-coated surface, individual cerebral cortical cells attached to the basal surface isolatedly like the Hyp-POA cells. Basal cell proliferation was very slow, similar to that on glass

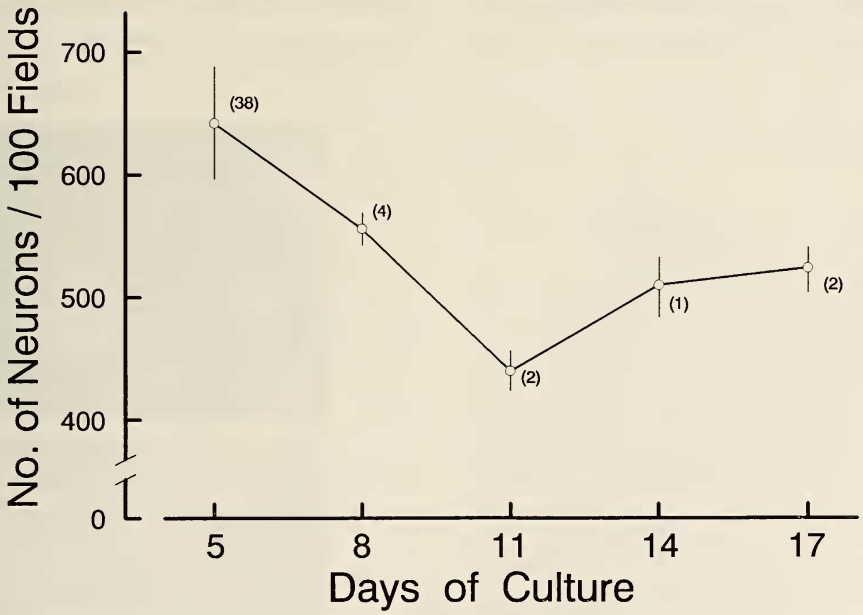

FIG. 4. The number of neurons in 100 microscopic fields of Hyp-POA cells cultured in serum-free medium on glass surface as a function of culture age. Vertical bars depict the standard errors of the means. The values in parentheses indicate the mean numbers of aggregates in 100 microscopic fields. Each value was obtained from triplicate cultures.

FIG. 3. Phase-contrast photomicrographs (A-D, F-H) or Bodian-stained preparation (E) of cultured Hyp-POA or CC cells in serum-free medium. A: Hyp-POA cells on glass surface at 3 h of culture. B: Hyp-POA cells on poly-lysine-coated surface at 3 h of culture. C: Hyp-POA cells on grass surface at 14 days of culture. D: Hyp-POA cells on poly-lysine-coated surface at 14 days of culture. E: Bodian-stained preparation of Hyp-POA cells on glass surface at 17 days of culture. F: Cerebral cortical cells on glass surface at 11 days of culture, showing differentiated astrocyte-like cells. G: Cerebral cortical cell aggregates at 11 days of culture. H: Cerebral cortical cells on glass surface at 14 days of culture. Note the degeneration of fiber connections between two aggregates. Bar in H=100 μm applies to A-D and F-H. Bar in E=50 μm.

surface. In some regions of a coverslip, surviving neuron-like cells developed very fine networks which were similar to those observed by Romjin *et al.* [17] in their fetal rat cerebral cortical cell cultures in serum-free medium. These neuron-like cells began to degenerate between 8 and 11 days of culture. Differentiated astrocyte-like cells were encountered as in the cultures on glass surface.

Effects of hydrocortisone on neuronal survival in serum-free medium

The Hyp-POA cells were precultured for 2 days in serum-supplemented medium (DME/F12-S) on glass surface. After the preculture, the medium was replaced by DME/F12-F containing hydrocortisone at the concentration of 0 (control), 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, or 10^{-7} M in 0.007% vehicle ethanol. Cultures were maintained for 14 days and the cells on coverslips were fixed and stained. As the concentration of hydrocortisone increased, the density of basal cells decreased, and basal cells became more fibrous in shape (Fig. 5C). At a concentration greater than 10^{-9} M, strange cell clumps which were brilliant with smooth outlines under the phase-contrast microscope, were observed at 8–14 days (Fig. 5B). These clumps were in most cases constricted and drifted out into the medium. Although such clumps were also encountered in control cultures without hydrocortisone, they were less in number and never drifted out. The number of neurons gradually increased by hydrocortisone treatment up to the concentration of 10^{-10} M (at 10^{-10} M, 134.5% of the control value). At a concentration greater than 10^{-9} M, the number decreased (Fig. 5A). The concentration that effectively decreased the number of neurons was the same as that where the formation of cell clumps gegan to increase.

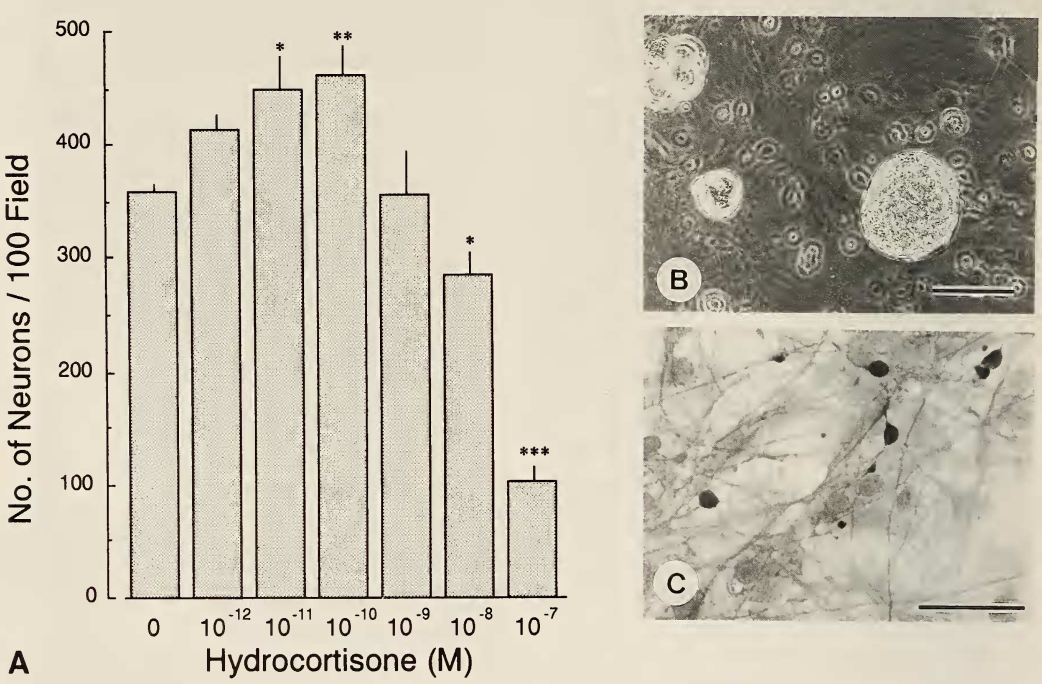

Fig. 5. Effects of hydrocortisone on the number of neurons (A) and the morphology of Hyp-POA cells (B and C) at 14 days of culture in serum-free medium. A: The number of neurons in 100 microscopic fields. Vertical bars depict the standard errors of the means (n=3). Significance of differences: (by analysis of variance) hydrocortisone-treated groups vs. control, * $P<0.05$, ** $P<0.01$, *** $P<0.001$. B: Phase-contrast photomicrograph of floating clumps treated with 10^{-7} M hydrocortisons at 11 days of culture. C: Bodian-stained preparation of cells treated with 10^{-7} M hydrocortisone at 14 days of culture. Note more numerous fibrous basal cells as compared to the cells in Fig. 3E. Bars=50 μm.

DISCUSSION

The increase of non-neuronal cell proliferation in serum-supplemented medium apparently disturbed the survival of neurons of the Hyp-POA. Godfrey *et al.* [4] reported that no electrical excitability or synaptic activity was elicited by brain cells in culture without treatment on non-neuronal cell proliferation. Such effect of non-neuronal cells appears to occur at relatively high plating density [18]. Most of the neurons which survived for 3 days were well maintained for the rest of the culture period and formed fine networks if cultured in serum-supplemented medium with AraC, which acts to inhibit the proliferation of non-neuronal cells, in the present study. Recently, it was reported that AraC killed cultured neurons derived from sympathetic ganglia by evoking active death processes intrinsic to the neurons [19, 21]. In addition, Brochovsky and Bradford [21] have studied whether or not AraC has any toxicity on postmitotic cells. They found that prolonged treatment with 10^{-5} M AraC prevented the survival of either neurons or astrocytes in the culture of whole brain cells from fetal rats, and stated that tetanus toxin-labeled cells, GFAP-positive cells and dopamine release all vanished after 14 days of culture. Hayashi and Patel [22] also reported that prolonged exposure to 10^{-5} M AraC was toxic to both the neurons and glial cells grown in chemically-defined medium. However, in the present study, 3-day exposure to 10^{-5} M AraC caused no remarkable death of neurons at later periods of culture. In additon to a low survival rate of neurons, serum-supplemented medium has some other problems. Honn *et al.* [3] reported that FBS contained 53 ± 19 ng/dl (mean $\pm$ S.D.) testosterone and 9.6 ± 2.7 μg/ml hydrocortisone, in addition to some amounts of T_4, GH and insulin. To exclude these effects of FBS, serum-free culture has been attempted by a number of investigators as in the present study.

At first, the culture of cells in serum-free medium from the very beginning was examined. DME/F12-F alone was unable to maintain the cells. Supplementation of BSA and/or precoating coverslips with FBS considerably improved the survival in DME/F12-F. However, the improvement was still inferior to the culture with serum-free culture after preculture in serum-supplemented medium. As regard to the role of BSA, Yamane [14, 15] has already indicated that it acts to protect the cells from exogenous and/or endogenous lytic factors, in addition to a role as a carrier of fatty acids and other metabolites. The present result that serum precoating helped both cell survival and fiber connection formation is in good accord with the finding of Faivre-Bauman *et al.* [23] that serum precoating was necessary for serum-free culture of the cells from mouse embryonic hypothalamus. Fibronectin has often been used as an attachment factor for neuronal cell cultures. However, Faivre-Bauman *et al.* [23] stated that so-called 'cold-insoluble globulin' (identical with fibronectin)-coating was less effective than serum precoating, suggesting that there should be other attachment factors in FBS. Hayman *et al.* [24] reported that the major attachment factor in FBS is vitronectin. Facilitation of cell attachment by serum procoating in the present study might be induced by vitronectin present in FBS.

Effects of substrates were examined in the present study. On glass surface, the cells of the Hyp-POA during preculture in serum-supplemented medium first formed aggregates. The aggregates gradually spread out in serum-free medium, as Faivre-Bauman *et al.* [23] found in cell culture of the fetal mouse hypothalamus. Non-neuronal cell proliferation was considerably suppressed and the number of neurons was almost constant during 11 to 17 days of culture. After 8 days, neural networks were observed. The cells could be maintained on glass surface in serum-free medium for at least 3 weeks. On poly-lysine-coated surface, the cells attached to the surface isolatedly, and after about 8 days of culture once-formed basal cell sheet contracted. The contraction might be induced if poly-lysine is degraded as time proceeds, because cell adhesiveness to the surface might become weaker than the inter- or intracellular tension, resulting in the detachment of the cells from the surface and induction of contraction. In fact, adhesiveness of the cells on poly-lysine-coated surface was very weak at 8 days, since basal cells were easily detached by pipettings.

Because of such basal cell contraction, poly-lysine-coated surface does not seem to be a suitable substrate for a long-term culture of the Hyp-POA cells in serum-free medium.

The morphology of cultured cerebral cortical cells was much different from that of the Hyp-POA cells. In cerebral cortical cell cultures, aggregate formation during preculture period was more apparent, and spreading out of non-neuronal cells from the aggregates was less obvious. The difference in aggregate formation was probably due to the difference in the adhesiveness of cels between the cerebral cortical and Hyp-POA cells. Less obvious spreading out of non-neuronal cells in cerebral cortical cell cultures might be due to the difference in the composition of cells of the aggregates. Differentiated astrocyte-like cells were more numerous in cerebral cortical cell cultures than in Hyp-POA cell cultures, suggesting Hyp-POA cell cultures contained more numerous undifferentiated cells which had higher migration activity. The regional difference in the astrocyte maturation was also reported by other investigators [25].

The cerebral cortical cells were not maintained for a long period either on glass or poly-lysine-coated surface. Some factors were probably insufficient or lacking in DME/F12-F for cerebral cortical cell cultures. Romijn *et al.* [17] proposed a serum-free medium for the cerebral cortical cells, consisting of 3 : 1 mixture of DME and F12 supplemented with double cocktail dose of N2 supplements, 0.1% BSA, 20 ng/ml T_3 and 0.2 μg/ml corticosterone. Some colonies of differentiated astrocyte-like cells were observed after 8 days in the present study. The shape of these cells was similar to that induced by glial maturation factor [26] or with dibutyryl-cycic AMP [27].

The beneficial role of corticoids suggested by Romijn *et al.* [17] was tested in the culture condition established in the present study. Hydrocortisone diminished proliferation of basal cells, and at higher doses (10^{-8} and 10^{-7} M) it induced morphological changes of the basal cells. It is well known that corticosteroids affect glial cells. Farooqui *et al.* [28] reported that hydrocortisone induced morphological changes of both rat glioma cell line C6 and normal hamster glial cell line NN cells and activated arylsulfatase and β-galactosidase, which are concerned with myelinogenesis at the concentration of 7.6×10^{-6} M. Montiel *et al.* [29] reported that 50 nM dexamethazone inhibited proliferation of C6 cells and stimulated activities of glycerol phosphate dehydrogenase and lactate dehydrogenase in C6 cells. Therefore, hydrocortisone might have acted on some aspects of glial differentiation. Strange clumps were formed by hydrocortisone, but the cellular constituent of such clumps was not examined in the present study. The number of neurons increased as the concentration of hydrocortisone increased up to 10^{-10} M, and at still higher concentrations it steadily decreased. Since hydrocortisone apparently affected non-neuronal cells, further study is needed for the analysis of direct effects of hydrocortisone on the survival of neurons. At any rate, the culture condition established in the present study may be useful for the investigation of other hormonal effects on the Hyp-POA.

Im summary, the culture in serum-free medium of the cells derived from the Hyp-POA in the present study showed that for the better survival of neurons preculture in serum-supplemented medium was necessary and glass surface was a better substrate than poly-lysine-coated surface, and that hydrocortisone at certain concentrations was beneficial for neuronal survival.

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